Plant Archives Vol. 19 No. 1, 2019 pp. 25-32 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

ASSAYS AVAILABLE FOR GENO TOXICITY ASSESSMENT OF AGROCHEMICALS : A REVIEW

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Abstract

Pesticides and herbicides are being used worldwide to meet the food needs of the growing population and tackle the negative effects of climate change in agriculture. These agrochemicals find its way through water into our water bodies and food consumed on a daily basis. The active principle is usually assessed for toxicity and safe limits of tolerance determined by toxicology regulatory bodies. However, lack of good farming practices leads to overuse and alarming levels of these chemicals to be introduced into our ecosystem affecting humans and all life. Even though the active principle may be tolerable there have reports where the formulations used to prepare agrochemicals inadvertently increase the potency and toxicity of the preparations. Assessment of genotoxicity of agrochemicals therefore becomes of utmost importance before introducing them into the fields. Toxicity effects may be immediate, leading to massive cell death and or organ dysfunction or lead to DNA damages and epigenetic changes which manifest symptoms over time in the lifetime of an individual. Changes in DNA also lead to horizontal transfer of toxic responses in progeny. This article discusses the various tests available for toxicity testing for agrochemicals and their ability in damage detection.

Key words: genotoxicity; agrochemicals; toxicity testing; epigenetics; DNA damage

Introduction

The use of pesticides and herbicides has increased exponentially worldwide due to the need to meet the food demands of growing population. The use of such chemicals has enhanced agricultural production with increased food production, reduction in vector borne diseases and longer shelf life of food products. However use of pesticides has its demerits with overwhelming evidence of risk to humans and other life forms. Genotoxicity assessment is essential to determine the safety of herbicides and pesticides and such assessments are gaining relevance in context of governmental legislations aiming to protect human and animal health.

Pesticides are used worldwide in the form of formulations which contains active principle and inert substances called as adjuvants. However, it is being increasingly known that pesticides can cause damages at genetic levels in living beings exposed to them. Several epidemiological studies done in the past have shown that occupational exposure to certain pesticides might be related to several kinds of cancer (IARC, 1991). Especially the risk of leukemia and multiple myeloma have significantly increased (Blair and White, 1981; Brown *et al.,* 1990) and also a rise in stomach, pancreatic, liver and bladder cancer have been associated with pesticide exposure (Burmeister 1981; Blair *et al.,* 1983; Stubbs *et al.,* 1984). Most testing of toxicity is carried out for the active principle in mammals for clearance into the market. A study of nine common pesticides demonstrated that the final formulations were several times more toxic than their active principles (Mesnage *et al.,* 2014). Besides, the active principle the adjuvants too are increasingly evidenced to be genotoxic. DNA damages have long term implications for human life and biodiversity as a whole.

Adjuvants used in glyphosate-based herbicides have found to be highly toxic to human cells. Ethoxylated

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adjuvants found in glyphosate-based herbicides were thousands of times more toxic than the active ingredient as such (Mesnage *et al.,* 2013). Therefore, such formulations are responsible for secondary side effects and in vivo long term toxicities which are not caused by the active ingredient alone (Seraline *et al.,* 2013). Glyphosate based herbicides have shown to be toxic and work as endocrine disruptors in human liver cell line HepG2 (Gasnier *et al.,* 2009). Acute exposure of adjuvants has been detrimental to human and mice (Bradberry *et al.,* 2004; Adam *et al.,*1997). Adjuvants toxicity are generally underestimated in pesticides as inert and not tested for long term regulatory experiments. Surprisingly studies show that they amplify the toxicity of their active principle all of the cases (Robin *et al.,* 2014). As the aim of adjuvants is to increase the solubility of the active ingredient and provide additional benefits like increased half-life, higher cellular uptake; enhancing it pesticidal activity (Marutani and Edirveerasingam, 2007) and thereby greater side-effects on health. They can even add to their own toxicity (Mesnage *et al.,* 2013). The differential toxicity between active principle of pesticides and their complete formulation now appears to be a general feature of pesticide toxicology. Assessment of genotoxicity becomes paramount before introducing these chemicals into the ecosystem and a series of tests are available based on the type of genotoxicity caused by the chemical. The assessment of genotoxicity is a necessary component for the safety assessment of substances, relevant in forming international policies and regulations aiming at the protection of human health and the environment. This article analyzes different types of genotoxicity and methods available to detect them. Figure 1 demonstrates a battery of tests based on the type of genotoxicity.

Ames test:

The Ames Test, also called as the bacterial reverse mutation test was developed by Professor Bruce Ames at UC-Berkeley in the 1970s. This is a fast and sensitive assay to determine the ability of a test compound to induce mutations in DNA (Hebert *et al.,* 2015). Ames test is based on the fact that any compound or chemical that is mutagenic in bacterial strains such as Salmonella typhimurium or Escherichia coli will also be consequently carcinogenic to test animals and on exposure to humans. Considering that it is a low cost, fast and easy to perform experiment, it has become a major tool for screening potential carcinogenic compounds (Maron and Ames, 1983). The most frequently used Ames cell lines of *Salmonella typhimurium* are TA 97, TA98, TA100, TA102, TA104, TA 1535, TA 1538 and *Escherichia coli* strains such as WP2, uvrA, pKM101 are used. As each strain is genetically non-identical, using several strains increases the possibility of detecting a mutagen. These Salmonella cell lines carry a mutant gene that restricts the cell from synthesizing histidine amino acid from compounds present in a standard bacterial media. The Escherichia strains likewise are tryptophan mutants. Therefore, these strains can survive only when these respective amino acids are provided in excess in the medium. However, when the strain is exposed to a mutagenic substance, the mutant gene can revert back to its functional state, making the bacterial cells capable of surviving in basic medium (Mortelmans and Zeiger, 2000; Mortelmans and Ricco, 2000). Such types of mutation are called as reversion and the mutant colonies that now begin to synthesize histidine/ tryptophan are called as revertant. Spontaneous reversion of several colonies is a natural phenomenon and fairly consistent with a particular strain. But in the presence of a mutagenic agent the number of revertant colonies per plate increases proportional to the increasing dose of the mutagenic compound (Maron *et al.,* 1981; Levin *et al.,* 1982). Many substances may not be mutagenic by themselves but may

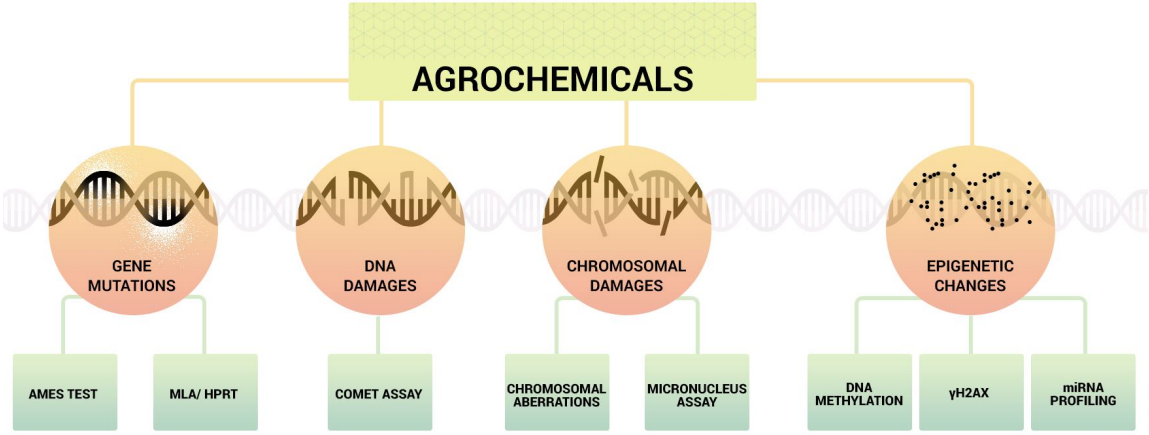

Fig. 1: Tests available to detect genotoxicity caused by agrochemicals.

be converted to mutagenic substances by metabolism in Liver. The by-products of metabolism in such cases may be carcinogenic. As bacterial systems do not have a metabolic system S9 microsomal fraction constituting rat or hamster liver enzymes are used to promote metabolic conversion of the test substance. Over the years the value of the test has been recognized by scientific community as well as regulatory bodies with this test being used worldwide as an initial screening step to detect mutagenic potential of compounds. The Ames test has been able to detect 90% of the known carcinogens and is financially viable, FDA recognized test. Therefore, it has been chosen as first choice for preliminary investigation of mutagenicity and carcinogenicity (Barbezan *et al.,* 2017).

MLA- Mouse Lymphoma Assay

MLA testing is designed to predict risk assessment before performing in vivo testing. The test utilizes mouse lymphoma thymidine kinase tk (+/-) cells and determines mutagenic and clastogenic effects of chemicals at the thymidine kinase locus by measuring resistance to a nucleoside analogue triflurothymidine (DOH, 2000). HPRT Testing is similar to MLA testing and determines clastogenic effect on HPRT locus present on X chromosome in mammalian cell line (OECD, 1997). In HPRT, mutations that make the HPRT gene dysfunctional are detected by positive selection using a toxic analogue. The HPRT mutants are selected as viable colonies. Since bacterial cells do not possess genome and metabolic machinery akin to mammalian cell systems (De Marini *et al.,*1989); HPRT test takes a upper hand to MLA testing. However, there are some disadvantages of using mammalian cells *in vitro* that is higher sensitivity and lower specificality with respect to mammalian genotoxicity. HPRT Assay was found to be negative for glyphosate even in high dosage levels with and without metabolic activation (Williams *et al.,* 2000). Therefore, a series of assays are generally used to understand mode of action, toxicity potential and to extrapolate to in vivo situation. The MLA assay is one of the most commonly used assays (Johnson GE, 2012). TK assay when performed using mouse lymphoma cell line such as L5178Y, the assay is called MLA (Moore *et al.,* 2000).

Comet Assays

Comet assays detects DNA strand breaks in cells and are referred to as Single cell- gel Electrophoresis Assay. As the name suggests, the damaged DNA spreads out on electrophoresis in the shape of a comet and the size of the comet tail increases in a dose- related manner of the toxic chemical substance. In this assay after exposure to test chemical a cell suspension is prepared and embedded onto agarose set on a microscopic slide. This allows to assess individual cells. The cells are then exposed to detergent and high salt solutions leading to cell lysis (Collins *et al.,* 2008; Azqueta *et al.,* 2014). Lysis allows the breakdown of cellular and nuclear membrane, allowing the DNA to spread out as a nucleoid (Azqueta *et al.,* 2011b). When assigned to electrophoresis, the DNA fragments will migrate towards anode, forming Comet shaped structure. The amount of strand breaks is proportional to the amount of DNA in the tail of the comet with respect to the comet head (Hovhannisyan, 2010). The slides can be visualised after staining with fluorescent dyes or silver stains under appropriate microscope. Data analysis requires a visual scoring of the head and tail from 0 to 4 according to the level of DNA damage, or computer-based image analysis such as caslab software can be used. This software allows quantification of several comet parameters such as tail extension movement or Olive tail movement and the percentage of tail DNA (Azqueta *et al.,* 2011b). Best scoring approaches are still debatable (Azqueta *et al.,* 2011a) but this technique allows robust data collection and statistical analyses. In a study to understand the genotoxic effect of fungicides, blood samples from 210 farmers exposed to a day of intense spraying with fungicides such as carbamates, organophospates and pyrethroids were collected. Comet Assay was carried out for this group as well as 50-member control group. Workers who were positive for DNA damages were assessed again after period of 6 months. Significant DNA damages were found in freshly exposed workers and followed up cases (Kaur *et al.,* 2011). In another study of bio monitoring of agricultural workers exposed to pesticides showed significant increase in tail migration of DNA (Carbajal-Lopez *et al.,* 2016). Comet Assay has become a simple and versatile technique to detect DNA damages.

Chromosomal Aberrations

Chromosomal Aberrations are structural alterations in the chromosome formed from double strand breaks. It has been observed that double strand breaks can be caused by exposure to toxic levels of non-mutagenic (Ames test negative) non-carcinogens (Storer *et al.,* 1996). This assay involves cytogenetic analyses from blood samples. Blood around 5 ml is collected in Vacutainer tubes and cultured for 52 hours in RPMI / Ham sF10 medium supplemented with mitogen phytohaemagglutinin and fetal bovine serum. Two hours prior to harvesting spindle block chemical colchicine is added. The cell division is now blocked at metaphase stage. The lymphocytes are collected by centrifugation and exposed to hypotonic solution of KCl. Fixation is carried out with methanol and acetic solution (3:1) and

slides are prepared by dropping the cell suspension onto a slide allowing breakdown of membranes and spreading of the chromosomes. The slides can be stained by Giemsa and observed for chromosomal aberrations (Patel *et al.,* 2017). In a study carried out at Jujuy, Argentina, 76 agricultural workers were tested for chromosomal aberrations using their lymphocytes. Significant increases in chromosomal aberrations were observed in the pesticide exposed workers (Bianco, 2017). Studies highlight to the clastogenic and DNA damaging effects of pesticides and insist on the need for biosecurity measures for occupational exposure.

Micronucleus Assay

Micronucleus assay has become one of the most widely used genotoxicity tests. This test is highlighted as the most significant for hazard identification and risk assessment by detecting the induction of chromosomal breaks (Hayashi, 2016). The fragments of DNA created by toxic chemicals, in a cell doesn't get attached to spindle apparatus during cell division and remain as secondary much smaller nuclei, which can be visualized distinctly on cytogenetic analysis. Atrazine is a triazinic herbicide and has been extensively used in rural agricultural areas. This also leads to high concentration of these agrochemicals washed into the nearby fresh water bodies. In a genotoxicity study carried out using *Oreochromis niloticus* as test animal, significant rate of micronuclei and nuclear abnormalities were detected on exposure to varying concentrations of Atrazine (Ventura *et al.,* 2008)

Epigenetic alterations and testing methods.

Epigenetic alterations include altered DNA methylation, histone modifications, non-coding RNA and chromatin remodelling (Tommasi *et al.,* 2014; Portela and Esteller, 2010). Several studies over the years have strongly pointed on the correlation between epigenetic changes and diseases like cancer (Hou *et al.,* 2012). Moreover, epigenetic changes have also been included in the list of key characteristics of human carcinogenesis (Chappel, 2016). DNA methylation is the most prominent and most studied phenomenon in many types of cancers. Environmental exposures of agrochemicals have neurotoxic effects and have been evidenced in the pathogenesis of neuro diseases such as Parkinson's. Several reports have shown overwhelming evidence of agrochemicals exposure in the development of Parkinson's disease (Corringan *et al.,* 2000; Costello *et al.,* 2009). Paraquat is a herbicide with chemical name 1. 10-dimethyl -4, 40-bipyridium is also a structural analogue to another neurotoxin 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) leading to parkinsons's (Hertzman *et al.,* 1990; Li *et al.,* 2005; Liou *et al.,* 1997; Peng *et al.,* 2005). Culture model studies on Parkinson's disease have also shown that paraquat caused histone acetylation (Song *et al.,* 2011).

DNA Methylation:

Epigenetic changes involve alterations in the genomic expression without involving changes in the DNA sequence. Even though the DNA sequence is unaltered the changes in epigenome is heritable and is carried forward in cell division cycles. DNA methylation is considered the most typical of the epigenetic changes. DNA Methylation refers to the methylation at CpG nucleotides also calledas CpG islands. The cytosine residue can be covalently modified by the addition of a methyl group at 5- Carbon position generating 5-methylcytosine (5-mC). This reaction is carried out by enzyme DNA methyltransferases, where the methyl group is transferred from S- adenosyl-L-methionine to a cytosine residue (Jurkowska *et al.,* 2011; Jeltsch *et al.,* 2014). DNA methylation can affect activity of one or both of paternal and maternal allele resulting in altered metabolism (Collotta *et al.,* 2013). A genome wide DNA Methylation study in Latino farm- workers and non-farm workers demonstrated a rise in DNA methylation at 36 CpG positions. The results involving 72 genes suggested widespread epigenetic changes in the occupationally exposed group (Howard *et al.,* 2016). DNA Methylation from exposure to diazinion to human hematopoietic K562 cells was demonstrated using Methylation Microarray that consisted of 27,578 individual CpG sites that included 14,000 genes. The study provided conclusive report that diazinon modifies gene promorter DNA methylation levels (Zhang *et al.,* 2012). DNA methylation of selected gene sequences can be investigated by Bisulphite Pyrosequencing Method. However, this assay helps to attain heterogeneous methylation patterns rather than high resolution at the level of single allele (Mikeska *et al.,* 2011). However there is a need to harmonize different methods for DNA methylation detection to generate tests with greater specificity and sensitivity to be applied as routine DNA methylation markers for testing (Mikeska *et al.,* 2014)

Phosphorylated (γ- H2AX):

Histone H2AX is one of the variants of the nuclear histone H2A. The phosphorylation of histone H2AX at γ- position was identified as an early event in DNA double strand breaks induced by ionizing radiations. The visualization and detection of γ- H2AX by flow cytometry helps to assess DNA damage, DNA repair and related DNA damage proteins.

Phototoxic chemicals are those compounds that have the ability to induce toxic effects when visible light or UV light (Ibuki and Toyooka, 2015). Such phototoxic chemicals can be found in foods, drugs, cosmetics, and other industrial substances (Epstein and Wintroub, 1985). γ- H2AX can be detected during S phase of cell cycle by flow cytometry and an increase in γ- H2AX can be associated with double strand breaks in the test cells (Tanaka *et al.,* 2007). Therefore it is possible to estimate the amount of γ- H2AX based on the cell cycle and in a time course manner in toxic compound exposure studies. Several studies also conclude that γ- H2AX measurement by flow cytometry is more sensitive analysis method of DNA damage than Comet Assays (Banath and Olive, 2003; Mah *et al.,* 2010). Dieldrin is a organochloride that is used as an pesticide and is a persistent organic pollutant. Studies have shown an increase in histone acetylation especially in core histone proteins and hyperacetylation plays an important role in neuron degeneration (Song *et al.,* 2010). The method of flow cytometry quantification of γ- H2AX is a fast and sensitive technique. Another method of detection is by immunofluorescence. γ- H2AX a phosphorylated protein is the first step in recruitment and localization of DNA repair proteins (Kuo and Yang, 2008). For immunofluorescence antibody against γ- H2AX can be used to localize γ- H2AX protein and detected by secondary antibodies (Nikolova *et al.,* 2014).

miRNA (microRNA) Alterations:

MicroRNAs are short oligonucleotides, 22 bp long that can negatively control gene transcription;post transcriptionally (Chuang and Jones, 2007). Alterations in the epigenome can also be attributed to the miRNA profile of exposed cells or organism thereby causing gene expression changes affecting cell metabolism (Collotta *et al.,* 2013). Variations in miRNA have been strongly implicated in cancer (Toyota *et al.,* 2008; Minor*et al.,* 2012). A study was performed to assess exposure of acaricide pesticide on carmine spider mite. Carmine spider mite is a crop pest and acaricides are routinely used for their control. However excessive use of acaricides has also seen the emergence of mite resistane against it. miRNA studies in carmine spider and comparison of different strains have highlighted how miRNA is involved in fenopathrin resistance (Zhang *et al.,* 2016). In another study involving paraquat exposure, changes in serum miRNA levels were observed in correlation to liver hepatotoxicity. The serum samples were analyzed by reverse transcription PCR and the results showed a positive correlation between alanine transferase (liver function biomarker) and serum miR-122 confirming the alterations in miRNA expression in response to agrochemicals (Ding *et al.,* 2012).

Conclusion

In this review we have highlighted the various assays available for different types of genotoxicity caused by agrochemicals. This review has focused on recent major advances in routine toxicity assays and also conventional practices. Conventional methods like chromosomal aberrations, Micronucleus and Comet are robust and sensitive techniques for environmental pollution monitoring. These traditional assays have been modified for automated data analysis and interpretation to increase sensitivity and specificity and also to provide high throughput capacity. Moreover, techniques involving epigenetic changes help to identify toxic substances that modify gene expression without causing DNA damages. These in the long run are important to assess risk of agrochemicals to humans and other organisms. Novel invitro epigenetic related methods are being developed which show promising results. They help to get a better understanding of the mode of action and allow extrapolation or prediction whenever animal studies or human exposure studies is not possible.

Most invitro studies involve cell lines and there is a possibility that we underestimate the toxicity of the chemicals and their by-products in the long term to human health. Several studies on human exposure have been positive for genotoxicity stressing the need for greater awareness of occupational hazard to agrochemicals. Importance of protective gear and equipment and safety protocols must be made aware to people in rural areas where majority of the farming takes place. Regular training of occupational workers and vigorous enforcement of safe farming practices is the need of the hour. There is no single assay solution to detect all genotoxic chemicals with different modes of action. Despite epigenetic marker assays being lucrative option, sensitivity of epigenetic assays needs to be assessed for clinical settings. On the other hand though invitro studies give advanced insights into level of toxicity potential of chemicals, animal studies are a must for safety assessment in today 'sscenario. A change is possible only when more research and development is put forward for data integration and finding convergent solutions in answer to the rigorous demands of regulatory testing. Conclusively, for long term sustenance; development of organic materials, limited and restricted use of agrochemicals is necessary for healthy living of mankind and ecosystem as a whole.

Acknowledgements

The authors are thankful to SRM Arts and Science College for providing the resources to write this review

paper.

Funding

This work received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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